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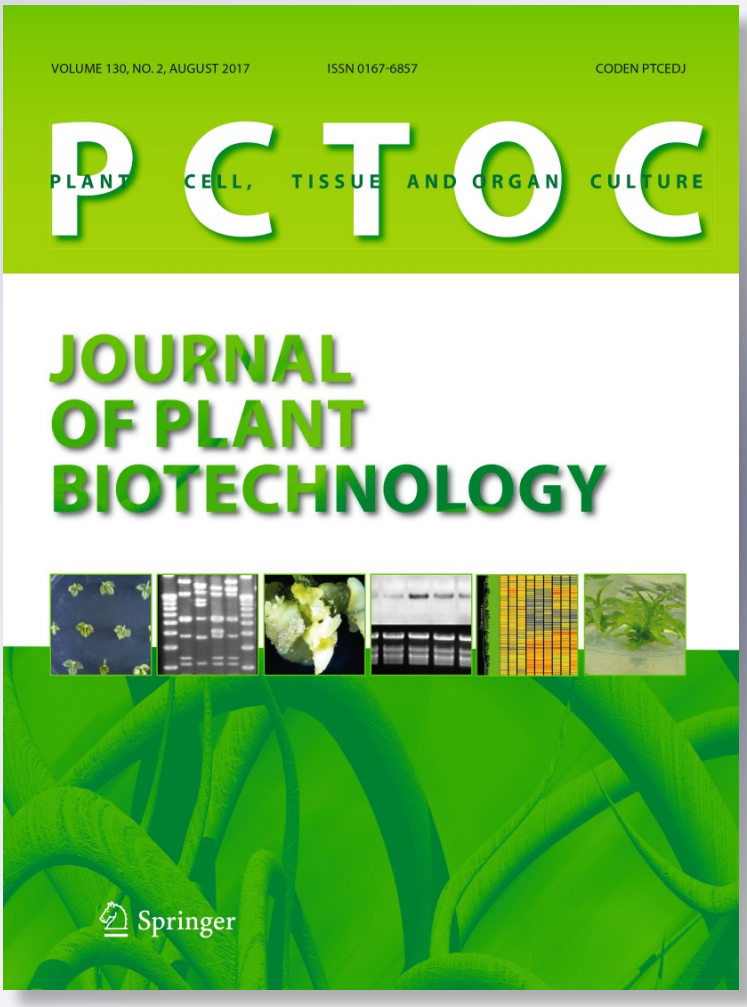

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ORIGINAL ARTICLE

Over-expression of a C3H-type zinc finger gene contributes to salt stress tolerance in transgenic broccoli plants

Ming Jiang[1] · Jing-Jing Jiang[2] · Li-Xiang Miao[3] · Cai-Ming He[1]

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Abstract C3H-type zinc finger proteins play important roles in plant growth, development, and stress responses. A C3H-type zinc finger gene, designated *BoC3H*, was isolated from broccoli (*Brassica oleracea* var. *italica*). The complete coding sequence of *BoC3H* was 1074 bp in length, encoding 357 amino acids with two CCCH motifs of C-X_7-C-X_5-C-X_3-H and C-X_5-C-X_4-C-X_3-H. The transcripts of *BoC3H* were profoundly induced by NaCl, and the highest expression level was observed at 18 h after treatment. Four broccoli lines over-expressing the *BoC3H* gene were obtained, and they exhibited higher germination rates, dry weight and chlorophyll content in response to salt stress as compared to those of wild type plants. Over-expression of *BoC3H* significantly decreased hydrogen peroxide (H_2O_2) level, relative electrical conductivity (REC) and malondialdehyde (MDA) contents, but dramatically increased free proline content, catalase, peroxidase and superoxide dismutase enzyme activities, resulting in less cell death in the leaves of transgenic plants. Taken together, our results suggest that BoC3H is likely to contribute to salt stress tolerance by regulating H_2O_2, REC, free proline, MDA and antioxidant enzyme levels in broccoli.

Keywords *Brassica oleracea* var. *italica* · C3H-type zinc finger · Salt stress · *BoC3H* · Over-expression

Introduction

Zinc finger proteins (ZFPs) are common, massive, and diverse biomacromolecules found in protists, fungi, plants and animals (Lee and Michel 2014). ZFPs involve in DNA replication, DNA recognition, RNA packaging, transcriptional activation, apoptosis regulation, protein folding and assembly, and lipid binding (Laity et al. 2001; Krishna et al. 2003). In plants, ZFPs are among the most abundant proteins, and are highly essential for various activities of plant growth, development, phytohormone signaling, and stress responses (Li et al. 2013). According to the number and order of conserved cysteine and histone residues, ZFPs are classified into several distinct types as C2H2, C3H, C2C2, C3HC4, C2HC5, and C3H2C3 (Li and Thomas 1998; Liu et al. 1999; Chen and Ni 2006). Among them, C3H-type ZFPs are characterized by a zinc finger motif containing three cysteine and one histone residues, and they consist of a large protein family (Bogamuwa and Jang 2014). A typical C3H protein is composed of one to six copies of CCCH zinc finger motifs, except for ZmC3H3 from *Zea mays*, which contained seven CCCH motifs (Peng et al. 2012).

In recent years, a small amount of C3H ZFP genes have been identified and functionally studied, and some of them are involved in abiotic or biotic stress responses (Yuan et al. 2015). The expression of *Arabidopsis thaliana ZFP1* was induced by salt stress, and its over-expression enhanced salt tolerance by maintaining ionic balance and limiting oxidative and osmotic stresses (Han et al. 2014). Over-expression of *GhTZF1* gene enhanced both drought and oxidative stress tolerance in *Gossypium hirsutum* (Zhou et al. 2014).

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OsTZF1 over-expressing *Oryza sativa* plants showed higher tolerance to high-salt and drought stresses (Jan et al. 2013). Another rice gene, *C3H12*, positively and quantitatively regulated resistance to *Xanthomonas oryzae* pv. *oryzae* (Deng et al. 2012). Two closely related C3H-type ZFP genes, *AtSZF1* and *AtSZF2*, played important roles in salt stress tolerance in *Arabidopsis* (Sun et al. 2007).

Plants are subjected to various environmental stresses, such as drought, nutrient deficiency, high temperature, freezing, disease, and salinity (Ramakrishna and Ravishankar 2011; Krasensky and Jonak 2012). Salt stress in soil or water is one of the major abiotic stresses, which affects plant growth, development and productivity, and, in extreme cases, causes plant death (Allakhverdiev et al. 2002). Excessive salt imposes an osmotic stress on plants, which can disrupt cellular structures as cortical microtubule organization and helical growth, and it can also impair physiological, biochemical and metabolic processes, resulting in cell disorder or injury (Krasensky and Jonak 2012; Shoji et al. 2006). Salt tolerance is regarded as a complex phenomenon, and the damage caused by excessive salt relies on plant species, variety, growth stage, and environmental factors (Yadav et al. 2011).

Broccoli (*Brassica oleracea* var. *italica*), a species of Cruciferae, is an important health-promoting vegetable crop for its various nutritive and functional molecules as vitamin C, dietary fiber, beta-carotene, diindolylmethane, carotenoid, glucoraphanin as well as selenium, and it can reduce the risk of getting a number of cancers (Vallejo et al. 2003). However, scarce information exists on C3H-type zinc finger genes involving in salinity (López-Berenguer et al. 2006; Smith et al. 2002). In the present study, a C3H zinc finger gene designated *BoC3H* was isolated from broccoli. Seed germination, dry weight, free proline content, relative electrical conductivity (REC), malondialdehyde (MDA) content, as well as antioxidant enzyme activities were determined and compared between the *BoC3H* over-expressing lines and the wild type (WT) broccoli plants.

Materials and methods

Plant materials

Seeds of broccoli (*B. oleracea* var. *italica*, QH80) were shown in plastic pots with mixture of peat moss and vermiculite (1:1, v/v) as substrate, and they were then cultured in an chamber at 25 °C with a 16 h light and 8 h dark cycle. At 2-leaf stage, seedlings were transplanted to containers filled with Hoagland nutrient solution, and were treated with 80 mM NaCl and equal amount of water as control. Leaf samples were collected at 0, 6, 12, 18, 24, and 30 h after treatment, and were stored in a −80 °C refrigerator for DNA and RNA isolation.

Cloning of *BoC3H* gene

Genomic DNA was isolated from 0.5 g fresh leaf samples using CTAB method (Doyle and Doyle 1987). Total RNA of leaves was extracted from each treated plants using Trizol reagent (Gibco BRL, USA). The first- and second-strand cDNA were synthesized using cDNA Synthesis Kit (Gibco BRL, USA). The complete coding sequence of *BoC3H* was amplified with primer pairs containing restriction enzyme sites (C3H3UP1: 5′-TCATCCCGGG ATGATGATCGGAGA-3′ and C3H3DN1: 5′-TACGAG CTCATGCATCACCAGTTCA-3′). Polymerase chain reaction (PCR) was prepared in a total volume of 20 μL containing 1 × PCR buffer (Promega, USA), 35 ng template DNA or cDNA, 0.2 μM of each primer, 0.8 U of *Taq* DNA polymerase (Promega, USA), 300 μM dNTP mixture, and 2.0 mM $MgCl_2$. PCR was performed using following profile: an initial denaturation at 94 °C for 5 min; template denaturation at 94 °C for 30 s, annealing at 57.9 °C for 45 s, and extension at 72 °C for 75 s for a total of 33 cycles; and a final extension at 72 °C for 10 min. PCR products were separated on 1% agarose gel containing ethidium bromide. The gel bands of interest were excised on a Gel Doc XR^+ System (BIO-Rad, UK), and were then purified using a QIAquick Gel Extraction Kit (QIAGEN, Germany). The purified fragments were cloned into pGEM-T EASY vector (Promega, UK), and the recombinant molecules were introduced into competent *Escherichia coli* strain DH5a for sequencing.

Sequence analysis

Seven homogeneous sequences of BoC3H were downloaded from NCBI (http://www.ncbi.nlm.nih.gov). Those homogeneous sequences were from *B. rapa* (accession number: XP_009102134.1), *B. napus* (CDY35891.1), *Capsella rubella* (XP_006297982.1), *A. thaliana* (NP_179571.1), *Arabis alpina* (KFK40348.1), *Eutrema salsugineum* (XP_006412868.1), *Camelina sativa* (XP_010433125.1), and *Tarenaya hassleriana* (XP_010540993.1). Multiple sequence alignments of BoC3H and its homogeneous proteins from other Cruciferae species were performed with ClusterX 1.81 (Thompson et al. 1997), and phylogenetic trees were constructed by Neighbor-joining method using Mega 3.1 (Kumar et al. 2004).

Expression of *BoC3H* gene

For qualitative Real-time PCR (qRT-PCR), two primer pairs were used to verify gene expression. The primer sequences were as follows: 5′-ACGAGTTTAAAGTCC GGCGA-3′/5′-AACTCACACGCGTCTCCTTT-3′ (amplicon size: 175 bp) for *BoC3H*, and 5′-ACGTGGACA TCAGGAAGGAC-3′/5′-GAACCACCGATCCAGACA CT-3′ (amplicon size: 175 bp) for the reference gene namely *BoActin*. qRT-PCR reactions were carried out in a LightCycler® 96 real-time PCR System (Roche, Switzerland) by using YBR FastStart Essential DNA Green Master Mix (Roche, Switzerland). The following reagents were pipetted into microcentrifuge tubes: 10 µl of Master Mix; 0.2 µl of each primer (20 µM), 3 µl of diluted cDNA, and 6.6 µl of ddH_2O. The PCR conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s. All qRT-PCR assays were performed in triplicate. Mean fold changes of *BoC3H* expression were normalized against *BoActin* by using equations described by Livak and Schmittgen (2001).

Expression vector construction and broccoli transformation

Purified PCR products were digested with both *Sma* I and *Sac* I, and were then cloned into pBI121 vector digested with the same enzymes. The recombinant vector was then introduced into *Agrobacterium tumefaciens* strain LBA4404. Murashige and Skoog (MS) supplemented with 0.02 mg/L of naphthaleneacetic acid (NAA), 4.0 mg/L of 6-benzylaminopurine (6-BA) and 5.0 mg/L of $AgNO_3$ was prepared for pre- and co-culture mediums. Shoot induction medium comprised MS plus 0.02 mg/L of NAA, 4.0 mg/L of 6-BA, 4.0 mg/L of $AgNO_3$ and 50.0 mg/L of kanamycin (Km). Tender stems of 18-day old seedlings were harvested, and dipped into 70% ethanol for 2 min, followed by immersion in 0.1% $HgCl_2$ for 8 min. Stem segments of 0.8 cm in length were placed on pre-culture medium for 3 d, and were then inoculated in a flask containing *A. tumefaciens* for 8 min. The explants were co-cultivated in the dark at 25 °C on the co-culture medium for 4 days, and transferred to shoot induction medium for 4.5 weeks. Green shoots were then cut and transferred to half strength MS medium containing 0.2 mg/L of NAA and 50.0 mg/L of Km for rooting, and the rooted plants were transplanted to sterilized soil for further study (Jiang et al. 2012).

PCR screening of transgenic plants and expression analysis

Genome DNA of broccoli lines was isolated using CTAB method. Primer pair C3H3UP3 (5′-CAACAAAGGGTA ATATCCGG-3′) and C3H3DN2 (5′-TCCGTAGCATCC GACTTGA-3′) was applied to amplify fragments with partial 35S promoter and *BoC3H* gene. DNA samples of recombinant plasmid (positive control), untransformed plant (negative control), and seven transgenic lines were employed as PCR templates. The total volume for each reaction was 20 µL, and reagents were added as follows: 35 ng template DNA, 20 pmol of each primer, 0.5 U of *Taq* DNA polymerase, 0.4 µM dNTP mixture, 50 mM Tris–HCl (pH 8.3), and 2.0 mM $MgCl_2$. The PCR amplification condition was as follows: 95 °C for 5 min; 33 cycles of 95 °C for 30 s, 55.0 °C for 45 s, and 72 °C for 60 s; followed by a final extension at 72 °C for 10 min. Four homozygous T_3 transgenic lines, namely B17-3-4, B26-5-2, B43-2-1 and B51-8-5, were chosen to verify the expression of the *BoC3H* gene using the same protocol and primer pairs as "Expression of *BoC3H* gene". For verifying *BoC3H* expression in over-expressing lines, leaves of 2-leaf stage seedling were collected. For measurements of expression pattern of *BoC3H* in over-expressing plants under different NaCl concentrations, leaf samples were collected at 0, 6, 12, 18, 24, and 30 h after treatments. RNA isolation, cDNA synthesis, and qRT-PCR were performed using the procedures and primers as described above.

Leaf disc assay and chlorophyll content determination

To estimate the effects of salt stress on mature broccoli leaves, leaf discs (1 cm in diameter) were prepared from fully expanded leaves of WT and the four T_3 transgenic lines. Leaf discs were floated on different concentrations of NaCl solutions (0, 40, 80, 120 mM) for 96 h. All treatments were repeated three times. For measurement of chlorophyll content, frozen leaf samples (0.5 g) were grinded with a pestle and morta into a fine powder in liquid nitrogen, and mixed with 8 mL 80% (v/v) acetone and kept overnight at 4 °C to extract chlorophyll. The chlorophyll was obtained after centrifugation at 9000 rpm for 10 min at 4 °C, and content of chlorophyll a and b was then measured by a spectrophotometer at wavelengths of 645 and 663 nm, respectively (Arnon 1949). Using the absorbance mean values, total chlorophyll content was calculated and expressed as µg/g fresh weight (FW).

Determination of dry weight, proline, MDA and REC

T_3 seeds of four transgenic lines, B17-3-4, B26-5-2, B43-2-1 and B51-8-5, together with WT seeds were used for germination test. The seeds were surface sterilized with 70% ethanol for 3 min and rinsed 4–5 times with sterilized distilled water, and were then sown in 90 mm × 15 mm petri dishes on filter paper beds with three replicates of 50 seeds, and each dish was irrigated with 3 mL of

respective NaCl solution (0, 40, 80, and 120 mM). The perti dishes were incubated in a 25 °C growth chamber for 7 days, and number of seedlings emerged were later counted for germination ratio calculation.

For dry weight, REC, proline and MDA determination, 2-leaf seedlings were transplanted to Hoagland nutrient solution supplemented with 0, 40, 80, and 120 mM NaCl. Each treatment was replicated three times. After 14 days of growth, plants were harvested for REC, proline and MDA determination. Free proline content was assayed according to the method of Bates et al. (1973). MDA content in leaf tissues was assayed by the method described by Saher et al. (2004). For REC measurement, six leaf discs of 6 mm diameter were taken from each sample and immersed in test tubes with 15 mL distilled water, and were then incubated in a 25 °C water bath for 24 h. The initial conductivity (EC_1) was measured using a DDS-11A conductivity meter. The test tubes were kept in a boiling water bath for 30 min, and cooled down to 25 °C for a final conductivity determination (EC_2). The relative EC value (%) was obtained by $EC_1/EC_2 \times 100\%$ (Apostolova et al. 2008). After 30 days of exposure to different salinity levels, whole plants were harvested and dried at 65 °C to constant weight, and dry weight was then measured.

Histochemical detection of $O_2^{\bullet -}$ and H_2O_2

For histochemical detection of $O_2^{\bullet -}$ and H_2O_2, NBT (nitroblue tetrazolium) and DAB (3, 3′-diaminobenzidine) *in situ* staining were respectively performed. Two-leaf stage seedlings were treated with 120 mM NaCl for 3 days, and the control plants were grown under the same conditions. Leaves from broccoli lines were incubated with freshly prepared NBT (2 mg/mL NBT in 50 mM sodium phosphate buffer, pH 7.5) or DAB (1 mg/mL DAB, pH 3.8) solution as described by Kumar et al. (2014).

Measurement of cell death by trypan blue staining

Trypan blue stock solution was prepared by mixing 10 mL of phenol, 10 mL of glycerol, 10 mL of lactic acid, 10 mL of distilled water, and 0.02 g of trypan blue. The stock solution was diluted with ethanol (96%; 1:2, v/v) to obtain trypan blue working solution (Pogány et al. 2009). After 3 d of exposure to 120 mM NaCl, leaves were excised and soaked immediately with ethanol/chloroform (3/1, vol/vol) mixtures for 24 h at room temperature. The colorless leaves were then stained with trypan blue working solution for 4 h, and cleared with saturated chloral hydrate solution for 30 min.

Protein measurement and enzyme assays

Fresh leaf (0.5 g) samples were frozen in liquid nitrogen and grounded to powder in mortars. The powder was then homogenized with 10 mL of 50 mM potassium phosphate buffer (pH 7.0). The homogenate was transferred to test tubes and centrifuged at 13,000 g for 20 min at 4 °C. The supernatant was used to measure catalase (CAT), peroxidase (POD) and superoxide dismutase (SOD) activities with three replicates for each assay. CAT activities were determined based on the procedure of Cakmak and Marschner (1992). POD activities were measured following the method of Maehly and Chance (1954). SOD activities were determined as described by Bayer and Fridovich (1987). Total protein concentrations were measured according to the method of Bradford (1976). All the enzyme activities were expressed as unit (U) / mg total protein. One unit of SOD activity was equivalent to the amount of enzyme that caused 50% inhibition of photochemical reduction of NBT at 560 nm. One unit of POD activity was defined as the amount of enzyme required to cause a change of 0.01 absorbance at 470 nm per minute. While one unit of CAT activity was equivalent to the amount of enzyme required to cause a decrease of 0.01 absorbance at 240 nm per minute.

All the data were statistically analyzed by using one-way analysis of variance (ANOVA) and Duncan's test at a significance level of $p = 0.05$.

Results

Identification and sequence analysis of *BoC3H*

The full-length cDNA and genomic DNA sequences of *BoC3H* were amplified. Sequencing results indicated that the complete coding sequence of *BoC3H* was 1074 bp in length, and no intron was identified within its genomic DNA sequence. The deduced protein sequence was comprised of 357 amino acids containing two C3H motifs, which lay respectively at residue sites of 123–141 and 158–173 (Fig. 1). According to residue numbers between three cysteines and one histidine, the two C3H motifs were described as C-X_7-C-X_5-C-X_3-H and C-X_5-C-X_4-C-X_3-H (X represents any amino acid), respectively.

BoC3H shared 99, 89, 81, 79, 77, 81, 79 and 76% identities with homologous sequences of *B. rapa, B. napus, C. rubella, T. hassleriana, E. salsugineum, A. thaliana, (A) alpina* and *C. sativa*, respectively. Multiple sequence alignments were generated by the ClustalX program. Both BoC3H and its homologous protein sequences contained two types of C3H motifs, namely C-X_7-C-X_5-C-X_3-H and C-X_5-C-X_4-C-X_3-H (Fig. 2). The two C3H motifs showed higher similarities than other sequence regions, and C3H

```
1   ATGATGATCGGAGAAACTCATCGTGCTTATCCAACGGTTCAGATTCCTCCATGGCCACTTAACGACGATCTAACGGCGGTTGATATTTACGGAAGTCCT
1    M  M  I  G  E  T  H  R  A  Y  P  T  V  Q  I  P  P  W  P  L  N  D  D  L  T  A  V  D  I  Y  G  S  P
100 GACGGCGGAAATAGCATGCTCGAGGCTTTGGCTGCGTTACAGTGTTATCTTCCGTCGAATGAGCCGGATCTGGATTCTGACCCGGAACTGTCCGGTCCA
34   D  G  G  N  S  M  L  E  A  L  A  A  L  Q  C  Y  L  P  S  N  E  P  D  L  D  S  D  P  E  L  S  G  P
199 GATTCGGCAATCGATACGTACTCATGTGATCATTTCCGGATGTACGAGTTTAAAGTCCGGCGATGCACTCGTGGCCGGAGCCACGACTGGACGGAGTGC
67   D  S  A  I  D  T  Y  S  C  D  H  F  R  M  Y  E  F  K  V  R  R  C  T  R  G  R  S  H  D  W  T  E  C
298 CCTTACGCTCATCCCGGAGAGAAAGCTCGCCGTCGAGATCCGAGGAAGTATCATTACTCCGGTACGGCGTGTCCTGAGTTTCGCAAAGGCGGTTGCAAA
100  P  Y  A  H  P  G  E  K  A  R  R  R  D  P  R  K  Y  H  Y  S  G  T  A  C  P  E  F  R  K  G  G  C  K
397 AAAGGAGACGCGTGTGAGTTCTCTCACGGCGTTTTCGAGTGCTGGCTTCATCCGGCGCGTTATCGGACTCAGCCGTGTAAAGACGGTGGTAACTGTCGC
133  K  G  D  A  C  E  F  S  H  G  V  F  E  C  W  L  H  P  A  R  Y  R  T  Q  P  C  K  D  G  G  N  C  R
496 CGTCGTGTTTGTTTCTTCGCTCATTCGCCGGATCAGATCAGGGTTTTGCCGAACCAAAGCCCCGATCGAGTTGATTCTTTCGACGGTGTGTCTCCGATT
166  R  R  V  C  F  F  A  H  S  P  D  Q  I  R  V  L  P  N  Q  S  P  D  R  V  D  S  F  D  G  V  S  P  I
595 CGTAGGGCGTTTCAGTTTTCGATTTCTCCGACCTCCGGTTCGCCGCCGGTGAGTCCACGAGACGACTCAGAGTCTTCTTCTTTATTTAATCGTTCTCTC
199  R  R  A  F  Q  F  S  I  S  P  T  S  G  S  P  P  V  S  P  R  D  D  S  E  S  S  S  L  F  N  R  S  L
694 GGGTCGGGTTCGGTAAACGACATCGTCGCGTGTATGAGGCATTTGCAGCTTAATAAAGTGAAGTCTCTTCCTTCCTCTTACAACAATCAAGTCGGATGC
232  G  S  G  S  V  N  D  I  V  A  C  M  R  H  L  Q  L  N  K  V  K  S  L  P  S  S  Y  N  N  Q  V  G  C
793 TACGGATCCGGGTTCGGATCGCCACGTGGATCACTCTTGGGTCCCGGGTTTCGGAGCCTACCAAATACCCCGTCCCGACCGGAGATTGGTTATATGGAC
265  Y  G  S  G  F  G  S  P  R  G  S  L  L  G  P  G  F  R  S  L  P  N  T  P  S  R  P  E  I  G  Y  M  D
892 ATTTGGGATAATGGTTTGGAGGAAGAACCGGCGATGGAACGGGTCGAGTCGGGTCGTGAACTGCGAGCGAAGATGTTCGAGAAGCTCAGCAAGGAAAAC
298  I  W  D  N  G  L  E  E  E  P  A  M  E  R  V  E  S  G  R  E  L  R  A  K  M  F  E  K  L  S  K  E  N
991 TGCATGGATCGGGTTGACCCGGATCCGTATCAGGGTTCAGGTGAAGCTCCTGATGTCGGGTGGGTCTCTGAACTGGTGATGTGA
331  C  M  D  R  V  D  P  D  P  Y  Q  G  S  G  E  A  P  D  V  G  W  V  S  E  L  V  M  *
```

Fig. 1 The coding sequence of *BoC3H* and its deduced amino acids. The two CCCH motifs are highlighted in shade. The stop codon TGA was marked with an *asterisk*

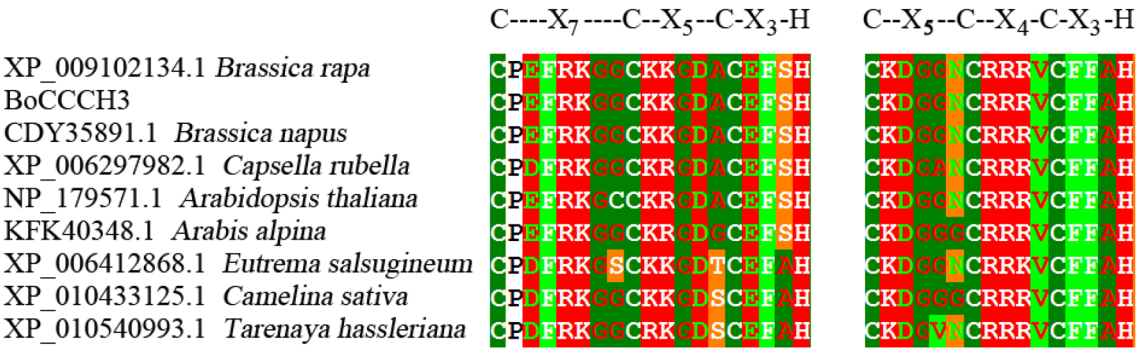

Fig. 2 Comparisons of C3H motifs between BoCCCH2 and its homologous sequences. Two motifs of C-X_7-C-X_5-C-X_3-H and C-X_5-C-X_4-C-X_3-H are depicted above the sequence alignment

motifs of BoC3H shared the same residues as those of *(B) rapa, B. napus* and *A. alpina*.

To investigate the evolutionary relationships of C3H between broccoli and its relative species, phylogenetic analysis was performed by using neighbour-joining method. Four well-supported clusters can be identified: cluster I for BoC3H and C3H ZFPs from other two *Brassica* species, *B. rapa* and *B. napus*; cluster II for *C. rubella* and *A. thaliana*; cluster III for *A. alpina*; and cluster IV for *T. hassleriana, E. salsugineum* and *C. sativa* (Fig. 3).

Induced expression of *BoC3H* in response to salt stress

qRT-PCR was performed by using gene-specific primer pairs, and the results were shown in Fig. 4. *BoC3H* transcripts were detected at all time-points in leaf mRNA when subjected to 80 mM NaCl, however, the expression levels were significant lower at 0 and 30 h. The expression of *BoC3H* increased significantly at 6 h and maintained its high level until 24 h, and the highest level of expression was observed at 18 h after exposure with a 3.21-fold increase as compared to 0 h. These results revealed that *BoC3H* was induced by salt stress.

Generation of *BoC3H* transgenic broccoli plants

To determine the possible biological function of the C3H-type zinc finger gene during salt tolerance, *BoC3H* gene driven by CaMV 35S promoter was transformed into broccoli WT plants, and a total of four over-expression T_3 transgenic lines, designated B17-3-4, B26-5-2, B43-2-1 and B51-8-5, were screened out of 58 regenerated lines using kanamycin, and they were later confirmed by PCR using C3H3UP3/C3H3DN2 primer pairs which amplified

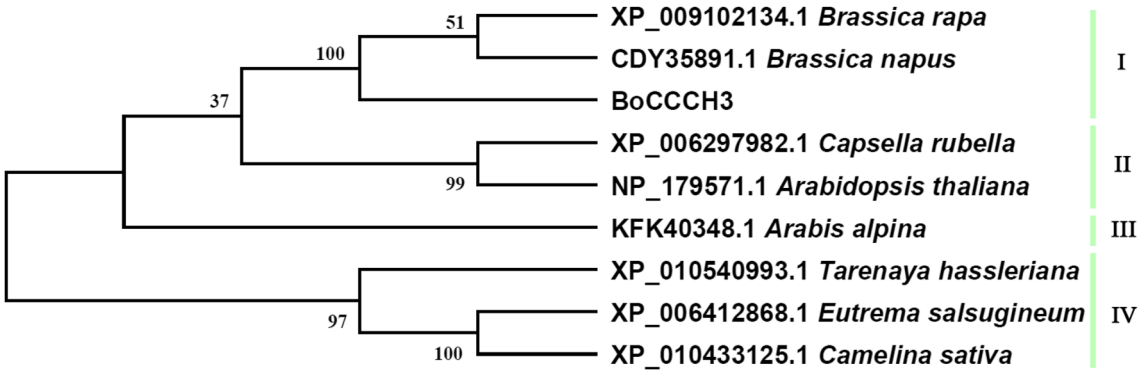

Fig. 3 A phylogenetic tree of BoC3H and its homologous sequences constructed by using neighbor-joining method. The numbers shown at the branches represent the bootstrap majority consensus values of 1000 replicates. I, II, III, and IV indicate four clusters

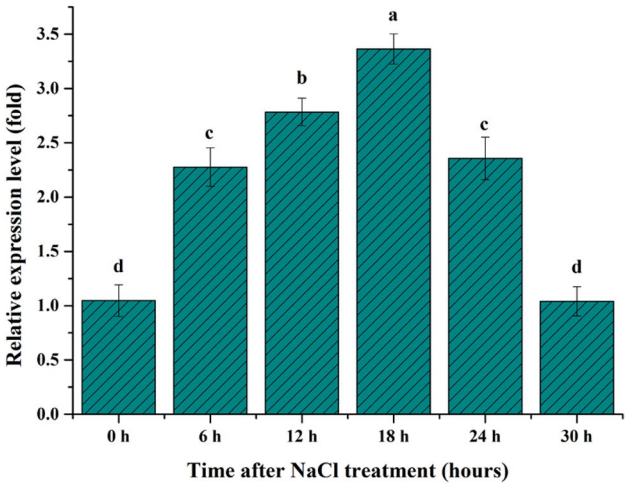

Fig. 4 Expression patterns of *BoC3H* in leaves in response to 80 mM NaCl. The value of each bar represents mean ± SD (n = 6) of three replicates, and *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests

a 1156 bp fragment. The amplified fragments with partial 35S promoter and *BoC3H* were detected in four transgenic lines as well as the recombinant plasmid, and there was no amplification observed in the untransformed plant and those negative plant lines (Fig. 5a). Expression analysis results indicated that all the four transgenic lines exhibited significantly higher expression levels, and the highest levels were observed in lines of B17-3-4 and B43-2-1 with an approximately mean fold change of 4.1 (Fig. 5b).

Transgenic broccoli plants exhibited higher levels of *BoC3H* expression

To investigate whether over-expression of *BoC3H* altered expression level in response to salt stress, qRT-PCR was performed to analyze gene expression profiles. qRT-PCR results indicated that the expression of *BoC3H* in over-expressing lines was up-regulated as compared to the wild type. At 0 mM of NaCl treatment, the expression levels of *BoC3H* in transgenic lines were higher than in the WT plant, with an increase of expression from 2.40- to 4.44-fold. All the over-expressing plants exhibited significantly higher expression levels subjected to 40–120 mM NaCl, and the fold changes ranged from 5.46 to 11.48 as compared to WT at 0 mM (Fig. 5c).

Comparison of seed germination rate and dry weight

Under 0 mM NaCl, all the five broccoli lines showed high seed germination percentage, and no statistically significant difference was found between WT and the four transgenic lines (Fig. 6). Germination rate was decreased with the increasing NaCl concentrations, and the lowest percentage was 71% for WT plant at 120 mM of NaCl, whereas the highest was found in four *BoC3H* over-expressing lines at NaCl concentration of 40 mM. All transgenic plants showed evident higher germination percentage than those of WT when treated with 40, 80 or 120 mM NaCl.

Dry weight under salt stress was determined after 30 days of treatment (Fig. 7). The dry weight decreased with the increasing of NaCl concentrations. No significant difference was observed between WT and the *BoC3H* over-expressing plants at 0 mM. However, evident difference in dry weight was found existed between WT and the transgenic lines when subjected to salt stress, and the dry weight of the transgenic lines was approximately 1.36–2.56 times higher than that of WT at 120 mM.

These results indicated broccoli plants over-expressing *BoC3H* contributed to seed germination and increased leaf dry weight when challenged with salt stress, demonstrating its positive role in salt tolerance.

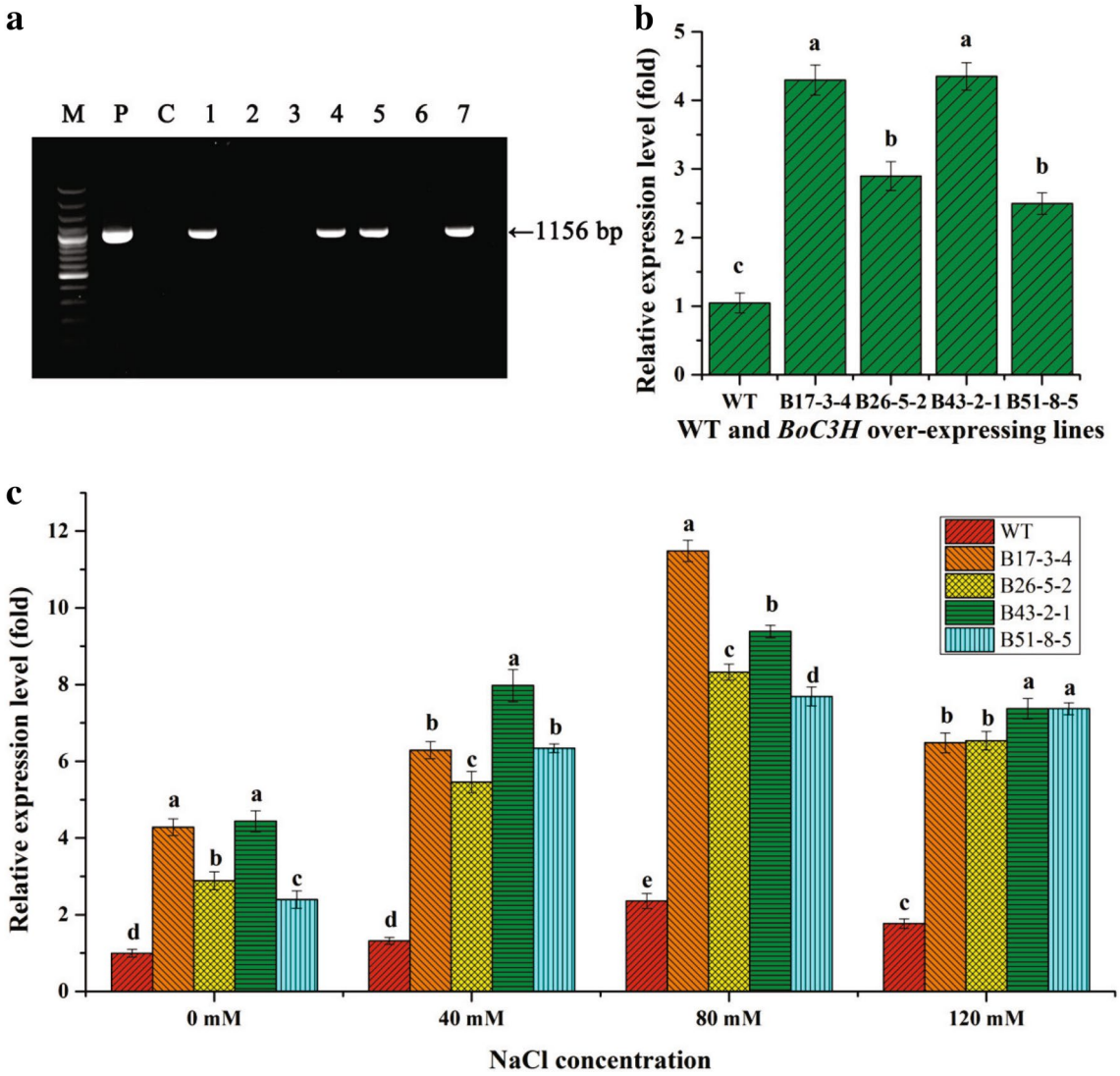

Fig. 5 PCR detection and expression analysis of *BoC3H* over-expressing lines. **a** Results of genomic PCR. **b** Expression analysis of *BoC3H* in leaves of over-expressing lines at two-leaf stage. **c** Expression of *BoC3H* in leaves of over-expressing lines in response to different concentrations of NaCl; the value of each bar represents mean ± SD of three replicates, and *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **c** Untransformed plant as negative control; P recombinant plasmid as positive control; *1*, *4*,*5* and *7* *BoC3H* over-expressing lines; *2, 3* and *6* negative lines; **M** 100 bp DNA ladder marker; **1156 bp** size of amplicon. **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

Effect of *BoC3H* over-expression on chlorophyll content and H_2O_2 accumulation

Leaf disc assay was performed to assess the tolerance of transgenic plants toward salt stress. Leaf discs of WT and *BoC3H* over-expressing plants were soaked in water solutions containing 0, 40, 80, 120 mM NaCl. After treatment for 4 days, the leaves from WT plants showed extensive yellowing, while the transgenic plants produced less chlorosis under the similar conditions, moreover, the leaf discs of both B43-2-1 and B51-8-5 remained green under concentration of 120 mM (Fig. 8a). These results were consistent with the chlorophyll content alteration. Under normal conditions, no difference in total chlorophyll content was observed between WT and the four transgenic lines. However, when subjected to salt stress, leaf chlorophyll content of WT plants declined more rapidly than the *BoC3H* over-expressing lines. In the presence of 120 mM NaCl, WT plants showed the lowest chlorophyll level, followed by lines of B17-3-4 and B26-5-2, and there was more than 2- to threefold chlorophyll loss in WT leaf discs (Fig. 8b).

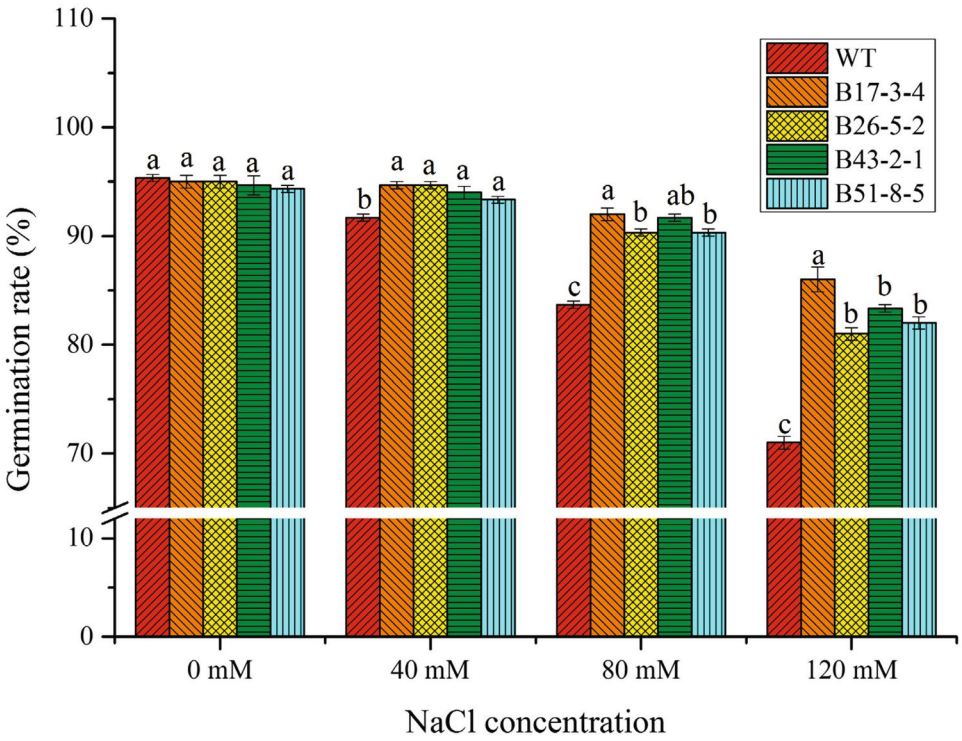

Fig. 6 Effect of salt stress on seed germination. The value of each bar represents mean ± SD (n = 6). Within each NaCl level of three replicates, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

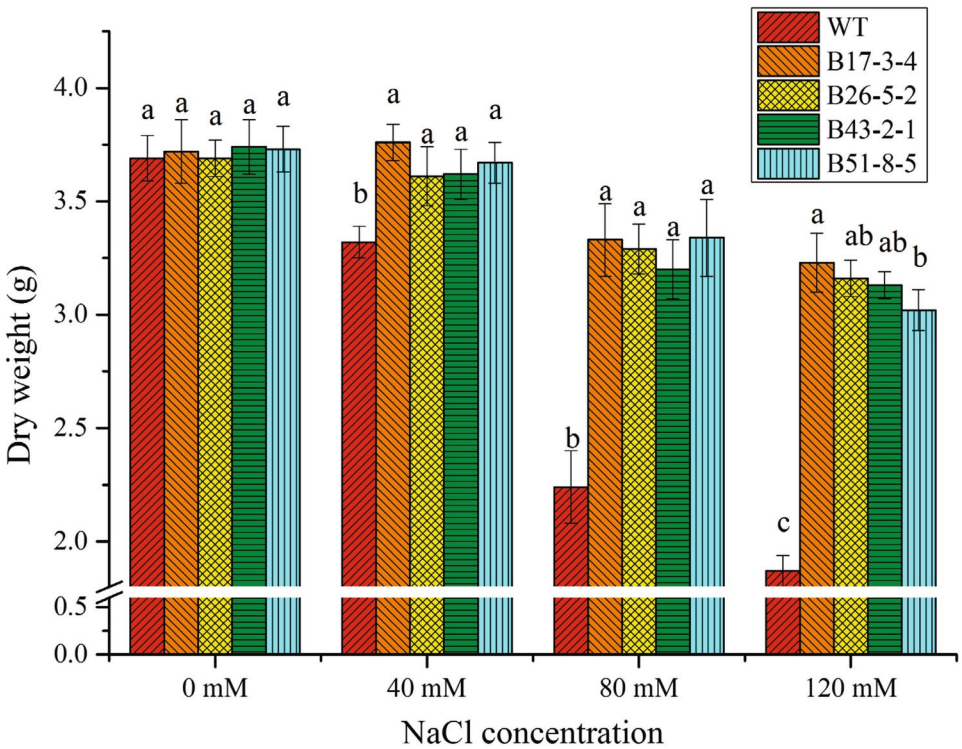

Fig. 7 Effect of salt stress on dry weight. The value of each *bar* represents mean ± SD of dry weight which was measured after 30 days of treatments under different NaCl concentrations. Within each NaCl level, *bars* with different *lowercase letters* indicate significant difference at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

The histochemical experiment of H_2O_2 in situ staining was performed by using DAB. Under normal conditions, less H_2O_2 accumulated in leaves of both WT and *BoC3H* over-expressing plants. However, when exposed to salt stress, brown coloration was highly accumulated in control leaves as compared to those of transgenic lines, indicating more H_2O_2 accumulation when challenged by NaCl (Fig. 9a). The results were later confirmed biochemically by estimating the H_2O_2 content, which in the leaves of the WT plants was 1.7–2.8 folds more as compared to those of

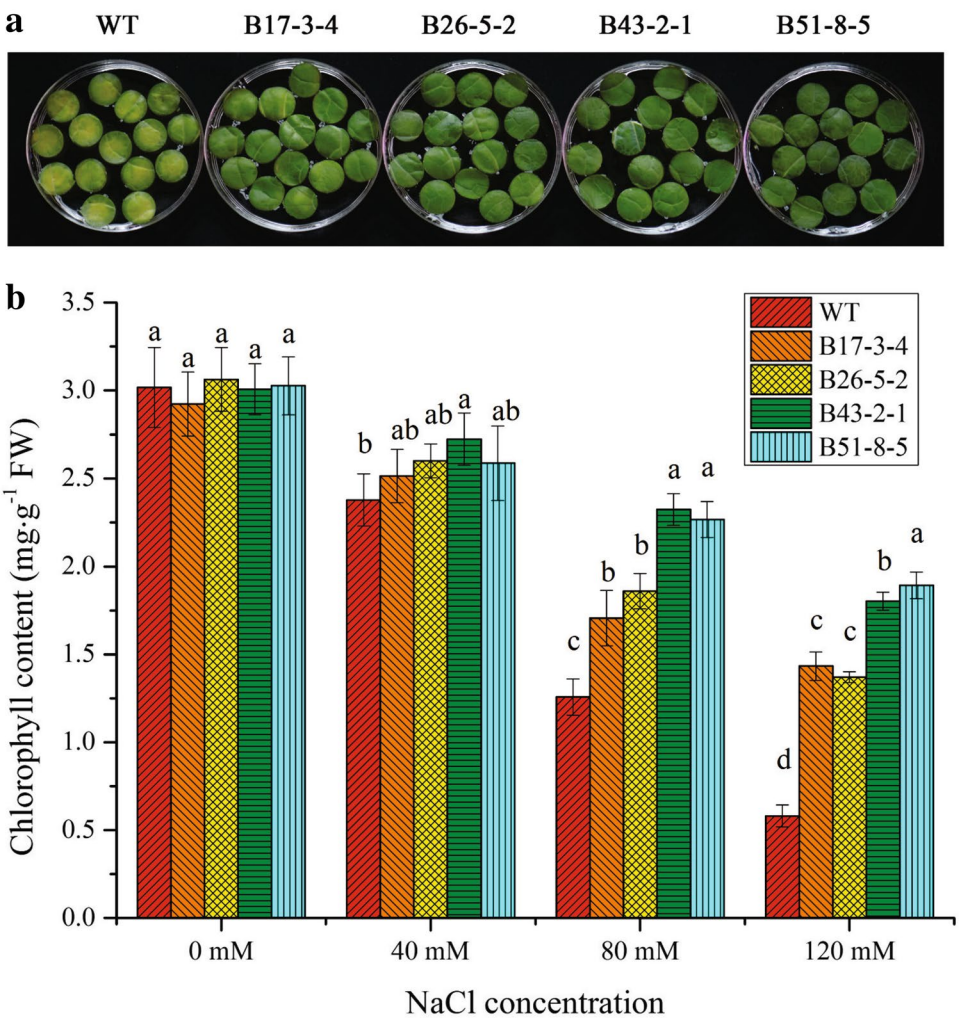

Fig. 8 Chlorophyll content of *BoC3H* transgenic broccoli plants under salt stress. **a** Leaf disc assay for salt stress at 120 mM NaCl; **b** the chlorophyll content retained in leaf discs. The value of each *bar* represents mean ± SD (n = 15). Within each NaCl level, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

transgenic lines (Fig. 9b). There was no significant difference in H_2O_2 accumulation between the transgenic lines in response to three NaCl concentrations except for 80 mM, in which higher H_2O_2 level was observed in B51-8-5 as compared to lines of B17-3-4 and B43-2-1.

These results revealed that over-expression of *BoC3H* helped decrease chlorophyll loss and quench H_2O_2 in transgenic plants, thus enhance their photosynthetic abilities and reduce toxicity.

Transgenic broccoli plants increased free proline level but decreased MDA contents under salt stress

Free proline accumulation was investigated in leaves of *BoC3H* over-expressing lines and WT plants treated by various concentrations of NaCl (Fig. 10). Proline content was increased in all lines at 40, 80, and 120 mM NaCl as compared to water treatment, and the highest values were observed at 120 mM. *BoC3H* over-expressing lines exhibited higher free proline accumulation than WT plants, and a maximal increase was found in B43-2-1 line. No significant difference was detected among transgenic lines at 0 mM, however, evident differences existed at 40, 80 and 120 mM NaCl, e.g. free proline in B51-8-5 line was significantly higher than in B17-3-4 and B43-2-1 lines at 40 mM, but it was evident lower as compared to B17-3-4 and B26-5-2 lines at 80 mM.

MDA content was determined in the leaf samples treated with different concentrations of NaCl (Fig. 11). Leaf MDA content in both *BoC3H* over-expressing plants and WT plants accumulated with the increase of NaCl levels, and the highest value was observed in WT plants at 120 mM. Significant differences were existed between the transgenic lines and WT plants, and all *BoC3H* over-expressing plants demonstrated evident lower MDA contents. No significant difference was present among transgenic lines at both 0 and 80 mM, however, evident differences were found between those at the other NaCl levels, e.g. B26-5-2 at 40 mM showed higher MDA than these in B17-3-4, B43-2-1 and B51-8-5 lines, while B17-3-4 and B26-5-2 at 120 mM demonstrated significant lower MDA content than in these in B43-2-1 and B51-8-5.

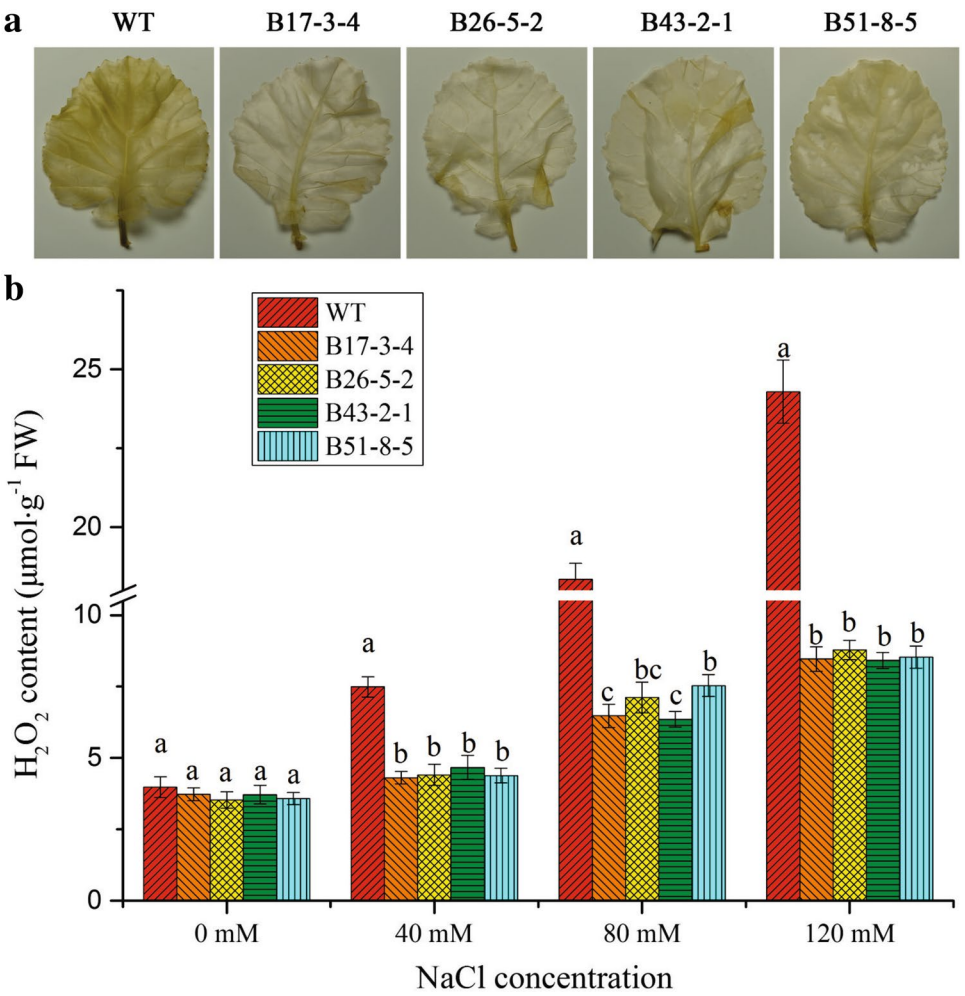

Fig. 9 Effect of salt stress on H_2O_2 content. **a** Histochemical detection of H_2O_2 in response to 120 mM NaCl by using DAB *in situ* staining; **b** H_2O_2 content in leaves of WT and the transgenic broccoli plants. The value of each *bar* represents mean ± SD (n = 6). Within each NaCl level, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

BoC3H over-expressing plants decreased REC in response to salt stress

The leaf samples of the *BoC3H* over-expressing lines and the WT plants treated with different NaCl concentrations were prepared for relative electrical conductivity (REC) measurement. REC in all leaf samples increased at different ratio with the increase of NaCl concentrations. Significant differences between the WT and the transgenic lines were detected with 0 mM NaCl, and the highest REC was observed in WT with a value of 21.24 ± 0.58. REC in the *BoC3H* over-expression lines were significant lower when treated with 40, 80 and 120 mM NaCl, and the lowest values were observed at 40 mM. While the WT plants treated with 40, 80 and 120 mM NaCl exhibited the highest REC (Fig. 12).

Transgenic broccoli plants showed higher antioxidant activities and less cell death

Histochemical experiments for $O_2^{\bullet -}$ in situ staining were carried out by using the NBT method. Under normal conditions, less blue substance accumulated in leaves of both control and *BoC3H* over-expressing plants. However, when exposed to salt stress, blue coloration was highly accumulated in WT leaves as compared to those of the transgenic plants (Fig. 13a). However, the transgenic lines showed less blue coloration, indicating less $O_2^{\bullet -}$ accumulation. To further characterize the phenotype of WT and the transgenic plants in response to salt stress, detached leaves were stained with trypan blue dye to visualize cell death. The leaves of the transgenic lines displayed less blue coloration relative to those of WT plants, indicating less cell death

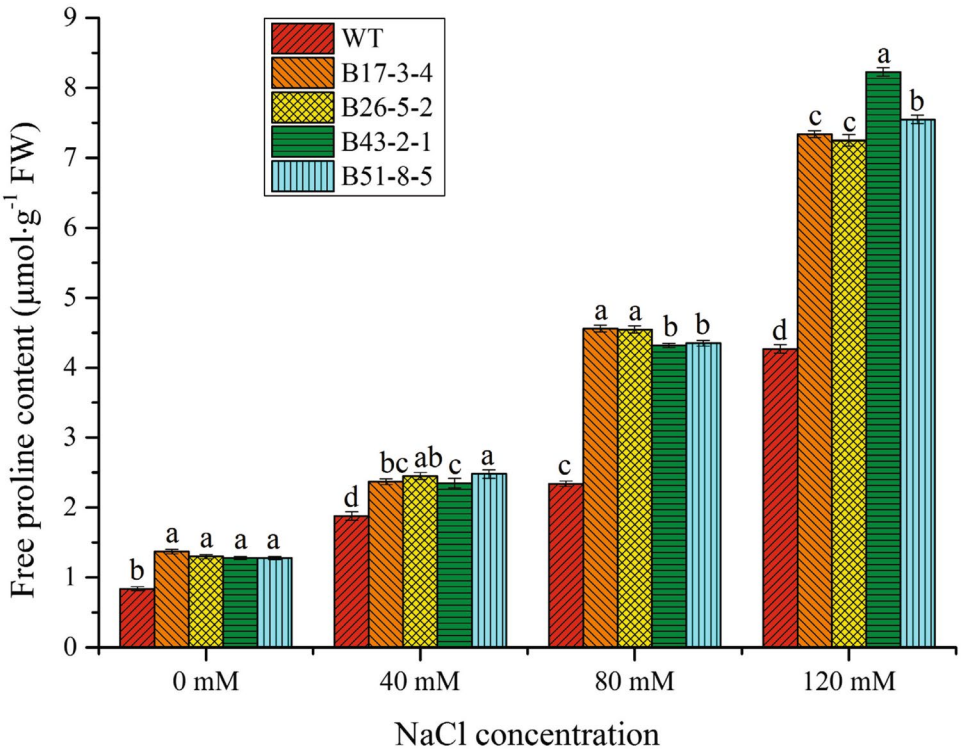

Fig. 10 Effect of salt stress on free proline content. The value of each *bar* represents mean ± SD (n = 6). Within each treatment, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

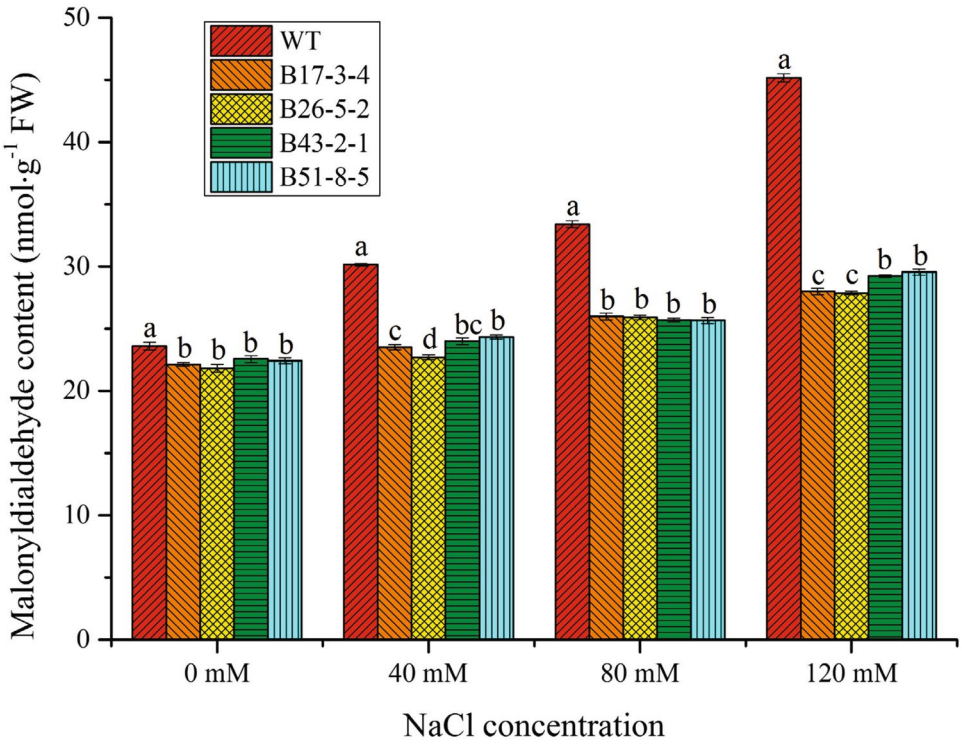

Fig. 11 Effect of salt stress on MDA content. The value of each *bar* represents mean ± SD (n = 6). Within each treatment, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

happened under salt stress (Fig. 13a). These results were corroborated by determining CAT, POD and SOD enzyme activities.

Significant differences were observed in both CAT and SOD activities between WT and the transgenic plants, however, there existed no difference in POD activity when treated with 0 mM NaCl (Fig. 13b-d). Increasing of salinity level increased leaf CAT, POD and SOD enzyme activities rapidly in transgenic lines, and there were significant differences as compared to WT plants in response to 40, 80

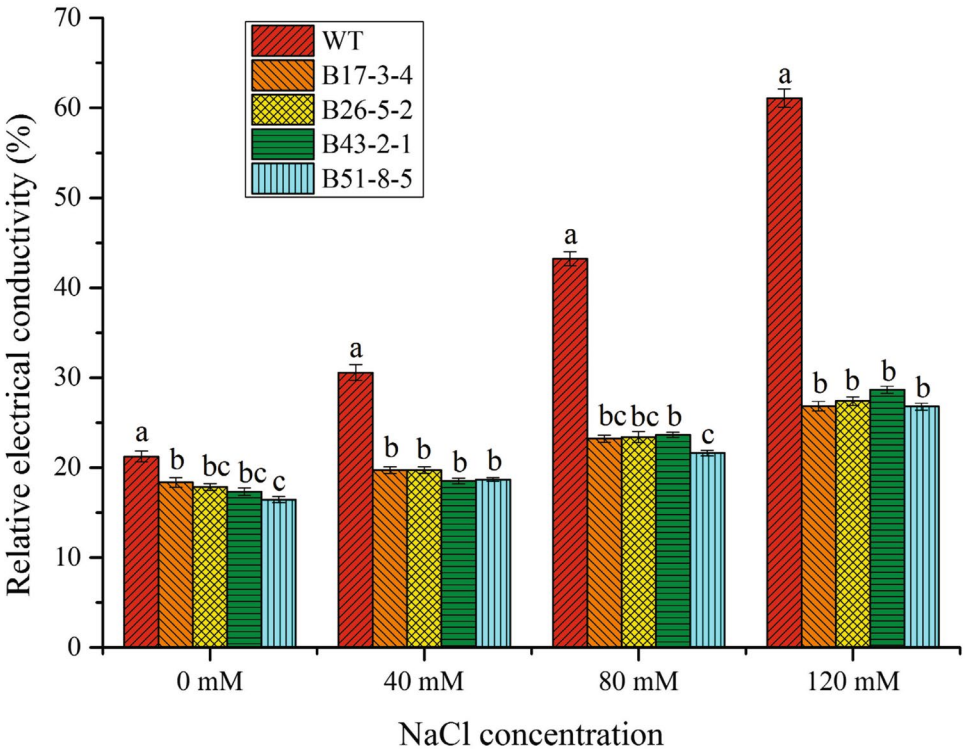

Fig. 12 Effect of salt stress on relative electrical conductivity. The value of each *bar* represents mean ± SD (n = 6). Within each treatment, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significance is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

and 120 mM NaCl. However, while in WT plants, the three enzyme activities increased slowly in both 40 and 80 mM NaCl, and then they decreased. The *BoC3H* over-expressing broccoli plants exhibited the highest enzyme activities at 120 mM NaCl, in which 2.4–3.2 folds more activity was observed as compared to those of WT plants.

These results revealed that over-expression of *BoC3H* contributed to quench radical toxins, and thus reduced reactive oxygen species (ROS) accumulation in transgenic plants, resulting in less cell death.

Discussion

C3H-type zinc finger proteins are defined as a zinc finger motif consisting of three cysteines and one histidine coordinated by a zinc cation (Bogamuwa and Jang 2014). C3H-type ZFPs are wildly distributed in eukaryotic organisms as yeast, protozoa, plants and humans, and have been implicated in multiple biological processes (Bogamuwa and Jang 2014; Thompson et al. 1996; Kramer et al. 2010). In plants, C3H-type ZFPs comprise a large family which can be divided into several subcategories or groups, and genome-wide analysis have revealed 68, 67, 68, 91, and 80 C3H zinc finger protein genes in *Arabidopsis, Oryza sativa, Zea mays, Populus trichocarpa*, and *Solanum lycopersicum*, respectively (Peng et al. 2012; Wang et al. 2008; Xu 2014). However, to our knowledge, no C3H-type ZFP gene has yet been identified from broccoli, and no research regarding gene functions involving in salt stress has been reported on this vegetable crop. In this study, a C3H-type ZFP gene, namely *BoC3H*, was isolated and characterized. The complete coding sequence of *BoC3H* was 1074 bp in length, and no intron was identified. Its homologous sequences of seven plants, *B. rapa, B. napus, C. rubella, T. hassleriana, E. salsugineum, A. thaliana, A. alpina* and *C. sativa*, contained no introns neither, and they shared high sequence similarity, suggesting that *BoC3H* might share similar biological function with these ZFP genes.

Based on the residue number between the four conserved amino acids in CCCH motif, C3H-type motifs are defined as C-X_{4-15}-C-X_{4-6}-C-X_3-H (Wang et al. 2008). The C-X_8-C-X_5-C-X_3-H and C-X_7-C-X_5-C-X_3-H are regarded as the most common C3H motif types. In *P. trichocarpa*, totally 211 C3H motifs are present in 91 gene members, and 96/76 of them belong to C-X_8-C-X_5-C-X_3-H/C-X_7-C-X_5-C-X_3-H types (Chai et al. 2012). Whereas in *Arabidopsis*, 78/43 out of 148 C3H motifs are C-X_8-C-X_5-C-X_3-H and C-X_7-C-X_5-C-X_3-H types, respectively (Wang et al. 2008). BoC3H contains two C3H motifs of C-X_7-C-X_5-C-X_3-H and C-X_5-C-X_4-C-X_3-H. The first one, as we know, is a very common type, and the second one, though less common than the first one, exists in *Clementine mandarin, P. trichocarpa, Arabidopsis, O. sativa* and *Z. mays* with motif numbers of 8, 16, 11, 9, and 9, respectively (Liu et al. 2014).

C3H-type ZFPs involve in various biotic and abiotic stress responses. Tomato *SlC3H43, SlC3H50, SlC3H76* and *SlC3H77* were up-regulated by salicylic acid

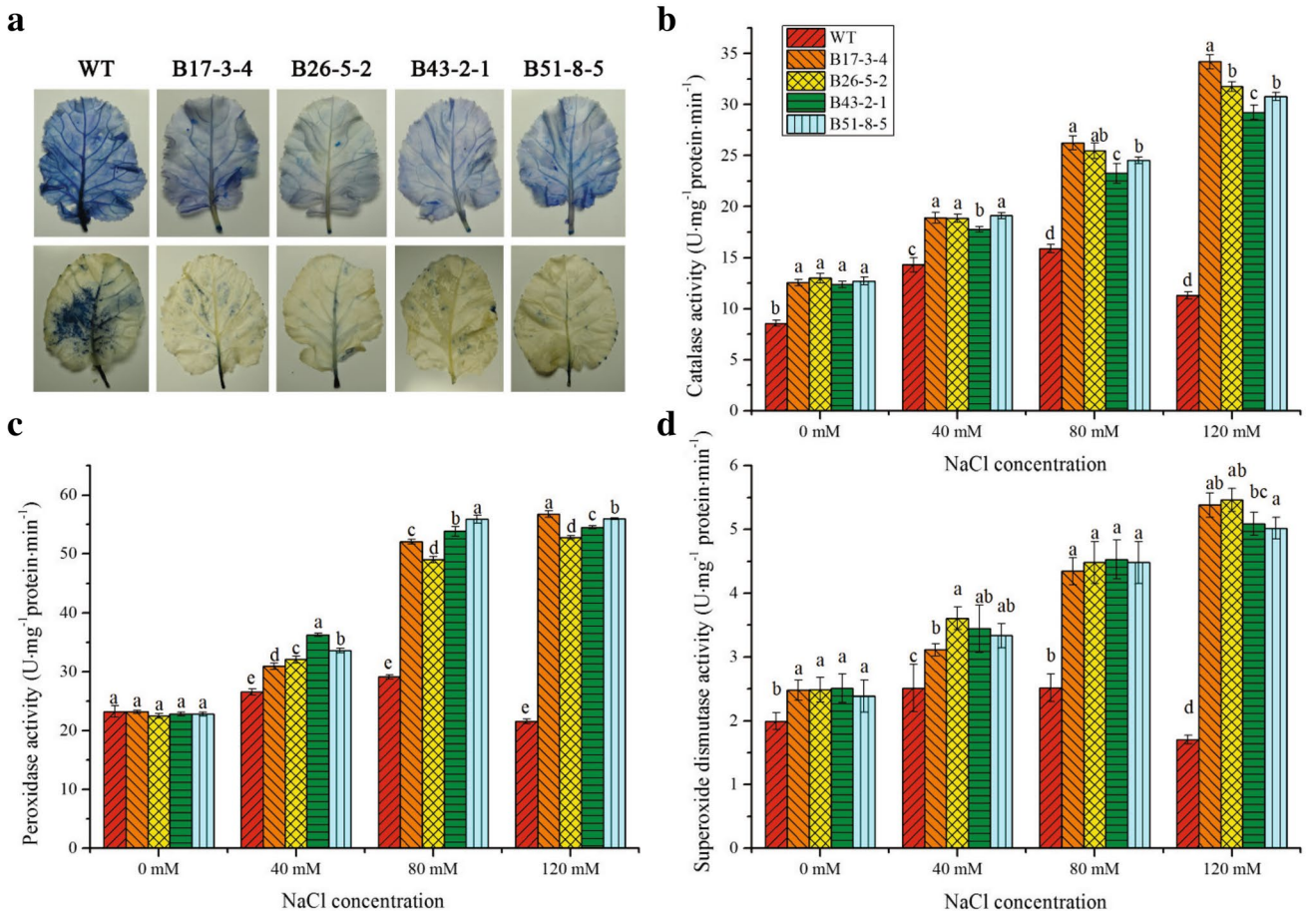

Fig. 13 Effect of salt stress on CAT, POD, and SOD content. **a** Detection of cell death after 3 d of exposure to 120 mM NaCl by using trypan dye staining (*top*) and histochemical detection of $O_2^{\bullet -}$ by using NBT *in situ* staining (*bottom*); **b, c** and **d** Enzyme activities of catalase, peroxidase and superoxide dismutase, respectively. The value of each *bar* represents mean ± SD (n = 6). Within each treatment, *bars* with different *lowercase letters* indicate significant differences at $p < 0.05$. The statistical significances is determined by Duncan's multiple comparison tests. **WT** untransformed plants; **B17-3-4, B26-5-2, B43-2-1** and **B51-8-5** the four *BoC3H* over-expressing T_3 lines

treatment, while *SlC3H49* and *SlC3H50* were down-regulated and up-regulated by 1-aminocyclopropane-1-carboxylate treatment (Xu 2014). In *Populus, PtC3H32, PtC3H33, PtC3H35, PtC3H38, PtC3H51* and *PtC3H72* were highly induced by drought stresses in roots, and further analysis revealed that these genes displayed different expression patterns between the two genotypes (Soligo and Carpaccio) of *Populus* (Chai et al. 2012). *GhZFP1* is isolated from a salt-induced cotton cDNA library, and its over-expression in tobacco enhanced tolerance to salt stress and resistance to *Rhizoctonia solani* (Guo et al. 2009). Expression of rice *OsTZF1* was induced by drought, high-salt stress, hydrogen peroxide, abscisic acid, methyl jasmonate, and salicylic acid, its over-expression exhibited improved tolerance to high-salt and drought stresses (Jan et al. 2013). In this study, *BoC3H* was induced by salt stress, and maintained higher expression levels from 6 to 24 h, indicating its possible role in salt stress response.

Seed germination and seedling stages are regarded as key developmental stages in plants, and they are very sensitive to salt stress, which affect germination as well as dry matter accumulation (Demir and Mavi 2008; Xu et al. 2011). Our results revealed that over-expressing of *BoC3H* increased both seed germination rate and dry weight, indicating its positive role in salinity tolerance. Salt stress causes chlorophyll reduction due to impaired biosynthesis or accelerated pigment degradation, and the extent of the reduction depends on salt tolerance of plant species (Ashraf and Harris 2013). *Broussonetia papyrifera* plants over-expressing *Festuca arundinacea FaDREB1* gene showed higher salt tolerance than WT, and higher leaf chlorophyll content was observed (Li et al. 2012). Rice plants over-expressing *OsOTS1*

increased salt tolerance and retained higher levels of chlorophyll as compared to control (Srivastava et al. 2016). In our report, transgenic broccoli plants showed less chlorophyll content loss under salinity stress, implying their higher photosynthetic capacity than those of WT plants.

Free proline, REC, and MDA are regarded as stress tolerance indictors, plants with rich free proline, lower REC and less MDA content have a positive correlation with their tolerance to various stresses (Zou et al. 2012). Proline accumulation is a common physiological response in plants when expose to biotic and abiotic stresses, it acts as an effective osmolyte stabilizing protein, cytosolic pH regulator, and hydroxyl radical scavenger (Verbruggen and Hermans 2008; John et al. 2016). In our study, free proline was significantly increased as compared to WT plants, and its overproduction was believed to play an adaptive role in salt stress tolerance. Salt stress causes cellular membrane damage, and results in an increase in electrical conductivity (Chen et al. 2013). REC in *BoC3H* over-expressing broccoli lines was significantly lower than in control plants, indicating that the transgenic plants suffered less membrane destruction under salt stress. MDA is regarded as a biomarker for lipid peroxidation, and it accumulates rapidly in response to stress. In our study, transgenic lines accumulate less MDA than those of WT plants, implying decreased permeability of plasma membranes in cells of *BoC3H* over-expressing lines.

ROS are chemically reactive chemical species containing oxygen, including superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), H_2O_2, singlet oxygen ($^1O_2$), and they are produced as normal products of plant cellular metabolism (Sharma et al. 2012). ROS play an important role as a messenger in normal cell signal transduction and cell cycling, and the extent of accumulation is determined by the antioxidative system (Sundaresan et al. 1995; Foyer and Noctor 2005). However, excessive ROS can contribute damage to cellular proteins, lipids and nucleic acids, leading to fatal structural and functional disorder (Zhang 2012). Biotic and abiotic stresses such as drought, salinity, chilling, metal toxicity, and pathogens attack generate harmful level of ROS (Sharma et al. 2012). Fortunately, plants have evolved effective antioxidant mechanisms to scavenge ROS, and in such a defense system SOD, CAT and POD act as important scavengers (Hajiboland and Hasani 2007). In our present study, SOD, CAT and POD in leaves of the transgenic plants increased significantly, and SOD converted more $O_2^{\bullet -}$ to H_2O_2, while CAT and POD reduced H_2O_2 to water, resulting in less $O_2^{\bullet -}$ and H_2O_2 accumulation in leaves (Tian et al. 2016). These results were correlated with histochemical staining of $O_2^{\bullet -}$ and H_2O_2 as well as cell apoptosis, in which less blue or brown coloration was observed, and moreover, less cell death symptoms were present in the transgenic plants, indicating less cellular injury produced by ROS (Pogány et al. 2009; Xu et al. 2014).

Conclusively, our results indicate that *BoC3H* is likely to contribute to salt stress tolerance by regulating H_2O_2, REC, free proline, MDA and antioxidant enzyme levels in broccoli, and the gene might be useful for molecular breeding of salt tolerant materials.

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Author contributions MJ and J-JJ conceived, designed and carried out the study. L-XM and C-MH carried out the study. MJ wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest All authors read and approved the manuscript. The authors declare that they have no conflict of interest.

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